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# The innate immune protein Nod2 binds directly to MDP, a bacterial cell wall fragment

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*innate immunity, nod-like-receptors, bacterial cell wall, Crohn's disease, muramyl dipeptide (MDP)*

Supporting Information Placeholder

**ABSTRACT:** Mammalian Nod2 is an intracellular protein that is implicated in the innate immune response to the bacterial cell wall and is associated with the development of Crohn's disease, Blau syndrome and gastrointestinal cancers. Nod2 is required for an immune response to muramyl dipeptide (MDP), an immunostimulatory fragment of bacterial cell wall, but it is not known if MDP binds directly to Nod2. We report the expression and purification of human Nod2 from insect cells. Using novel MDP-self-assembled monolayers (SAMs) we provide the first biochemical evidence for a direct, high affinity interaction between Nod2 and MDP.

The innate immune system is the body's first line of defense against invading pathogens<sup>1,2</sup>. This ancient system has evolved to exist in a symbiotic relationship with commensal bacteria and at the same time to recognize and destroy virulent bacteria<sup>1-3</sup>. Chronic inflammatory diseases such as asthma, rheumatoid arthritis, and Crohn's disease are thought to arise from an inappropriate innate immune response to bacteria<sup>4-9</sup>. Chronic inflammation has also been shown to lead to a variety of types of cancers including those affecting gastric, colon, and lung<sup>10</sup>.

Mammalian Nod2 is an intracellular protein that is involved in the signaling response to bacterial cell wall fragments<sup>11,12</sup>. Mutations in Nod2 correlate with the development of Crohn's disease, a chronic inflammatory disease of the gastrointestinal tract<sup>13-15</sup>. In order to generate the proper immunologic response, the Nod2 signaling pathway must recognize bacteria. The biochemical mechanism by which Nod2 detects bacteria is not known, but it has been proposed that Nod2 senses bacterial cell wall fragments directly. Nod2 could sense bacterial cell wall fragments through at least three mechanisms: (1) a direct interaction; (2) a mediated interaction; or (3) a signaling relay. The mechanism of activation remains unresolved because until now the proper tools to probe the mechanism have been unavailable.

Nod2 is essential for the cellular response to a small fragment of bacterial cell wall, muramyl dipeptide, consisting of one carbohydrate and two amino acids (**MDP-(D)**, **1**, Figure 1)<sup>12,16</sup>. MDP is found in both Gram-negative and Gram-positive bacteria. Cellular and *in vivo* assays have shown that when mammalian cells expressing Nod2 are treated with MDP, an inflammatory response is activated via the NF- $\kappa$ B and MAP

kinase pathways<sup>17-20</sup>. Moreover, the response is not observed if a diastereomer of **1**, (**MDP-(L)**, **2**, Figure 1), is used in the cellular assays. For this reason, MDP is often referred to in the literature as the "ligand" for Nod2 signaling<sup>21</sup>. However, there is no biochemical or biophysical data that demonstrates an interaction between the two molecules<sup>22</sup>. The purpose of our investigation was to determine if Nod2 and MDP interact *in vitro*. We developed an expression system and a biochemical assay using synthetic probes to investigate this question.

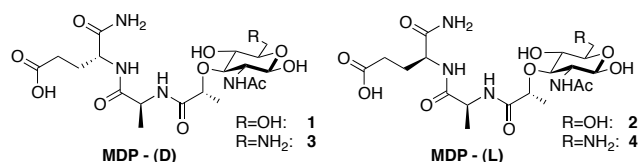


Figure 1: Muramyl Dipeptides: MDP-(D) is the biologically relevant isomer; MDP-(L) is a synthetic diastereomer of the compound found in Nature.

Human Nod2 is a large protein (1040 residues, 110 kD) with multiple domains: two N-terminal caspase recruitment domains (CARDs), a central nucleotide oligomerization domain (NOD) and a C-terminal leucine rich repeat (LRR) domain<sup>16</sup>. To determine if Nod2 interacts directly with MDP we first expressed a Flag-tagged version of Nod2 using baculovirus-infected Sf21 cells (Figure S1a in the Supporting Information) with a yield of 1 mg/L. CD spectroscopy and limited proteolysis experiments are consistent with Nod2 being a folded protein (Figure S1b and S1c in the Supporting Information).

With purified Nod2 in hand, a Surface Plasmon Resonance (SPR) assay was developed to assess binding to MDP. Initial attempts to develop a SPR assay with biotinylated-MDP<sup>23</sup> failed, as we observed significant non-specific binding of Nod2 to the streptavidin/biotin chip lacking MDP (Figure S2). In order to develop a SPR assay, we coupled 6-amino MDP (**3** & **4**, Figure 1) directly to the chip without the use of biotin. **3** & **4** are synthetic intermediates of the biotinylated -MDPs and have been shown to activate Nod2 in the appropriate manner<sup>23</sup>. Using methodology developed by Whitesides and co-workers, we prepared carboxy-terminated alkane thiol self assembled monolayers (SAMs) and then used on-chip NHS/EDC activation of the carboxylic acid (Figure 2)<sup>24</sup> to couple 6-amino-MDPs to the chip surface.

A typical SPR assay uses four sensor lanes on a single chip<sup>25</sup>. In the assay, we included two controls: (1) the iso-glutamine diastereomer of MDP (**4**, Figure 1) that does not activate the Nod2 pathway and (2) an ethanolamine-capped monolayer (Figure 2). A typical assay setup involved flowing Nod2 over each lane of the sensor chip and observing changes in resonance units (RU). The assay was robust and allowed the screening of a wide variety of conditions. There was lower background binding of Nod2 to the synthetic chip as compared to the biotin chip (Figure S2 and S3).

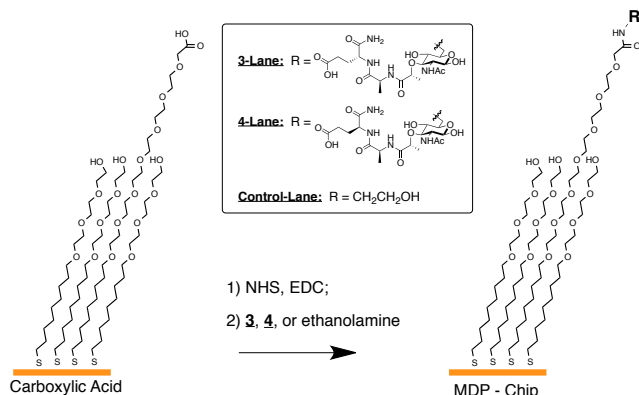


Figure 2: Synthesis of the MDP Chip: A mixed SAM consisting of 1% mole fraction of hexa(ethylene glycol)-carboxylic acid (EG)<sub>6</sub>CO<sub>2</sub>H-terminated thiol in tri(ethylene glycol) ((EG)<sub>3</sub>OH)-terminated thiol was prepared. The carboxylic acid groups were then activated with NHS and EDC to form the NHS ester. Displacement of the NHS ester with the amino group of MDP afforded the formation of an amide bond. Excess NHS esters were deactivated with ethanolamine.

Nod2 bound to MDP with high affinity (Figure 3). The biologically active MDP, **3-Lane**, bound to Nod2 with a  $K_D$  of 51 nM  $\pm$  18. Surprisingly, we found that Nod2 was able to bind to both isomers of MDP (Figure 3), as Nod2 bound to the **4-Lane** with a  $K_D$  of 150 nM  $\pm$  24, which is only slightly higher than the  $K_D$  observed for the **3-Lane**. Thus, the iso-glutamine stereochemistry is not a key recognition determinant. To demonstrate that binding of Nod2 to the MDP-chip is indeed specific, a competition study was performed. When Nod2 was pre-treated with either **3** or **4**, diminished binding to the chip was observed (Figure 4). Encouragingly, the competition data show the same trend as the on-chip data, i.e. the free D-isomer of MDP is a better competitor than the free L-isomer. These data suggest that Nod2 senses bacterial cell wall fragments by binding directly to them.

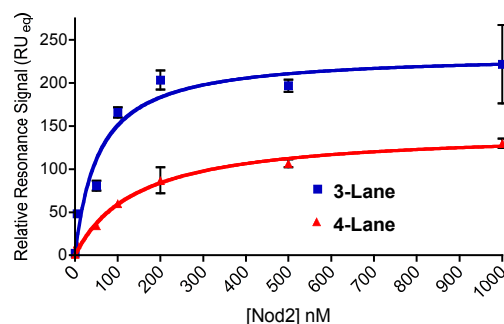


Figure 3: Nod2 binds to MDP: **3** and **4** bind to MDP with high affinities. Nod2 (pH 6.0) was applied to the MDP-Chip at varying concentrations at a rate of 3 mL min<sup>-1</sup>. After ten minutes, the

resonance signal was recorded, ethanolamine background was subtracted from the experimental signal and plotted. The data was fitted to a standard one site binding model by non-linear regression analysis using Prism 4 (GraphPad).

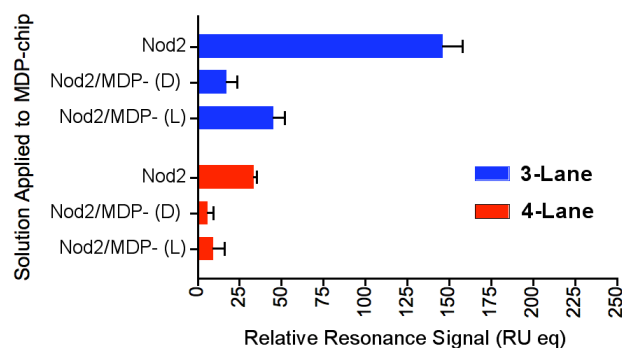


Figure 4: Binding of Nod2 to the MDP-chip is specific. Nod2 (pH 5.5, 0.5  $\mu$ M, either in the presence or absence of free MDP (1  $\mu$ M)) was applied to the MDP-Chip. The relative resonance signal was recorded after ten minutes.

At low concentrations the MDP isomers **2** and **4** do not activate the NF- $\kappa$ B response via Nod2 in cellular assays<sup>18,23</sup>. However, we show that MDP-(L) is able to activate the pathway at higher concentrations using the established cellular NF- $\kappa$ B luciferase reporter assay and transfected Nod2 (Figure 5). The NF- $\kappa$ B activation observed in the absence of transfected Nod2 DNA is the result of low levels of endogenous Nod2 in Hek293T cells<sup>26</sup>. The cellular assay results demonstrate that both isomers of MDP are able to activate the Nod2/NF- $\kappa$ B pathway, which is consistent with the binding data showing that Nod2 can bind one isomer better than the other. Cellular potencies often do not exactly match their *in vitro*  $K_D$ s<sup>27,28</sup>. We find the cellular activation parallels *in vitro* binding but at slightly lower potency.

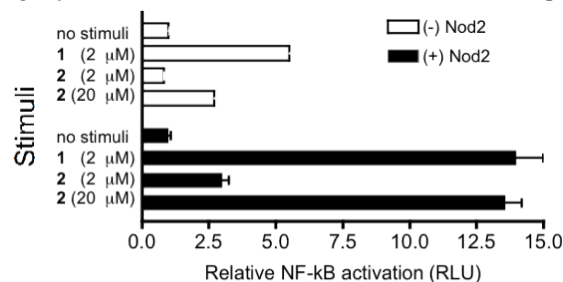


Figure 5: **2** (MDP - (L)) activates the Nod2/NF- $\kappa$ B pathway at higher concentrations. Hek293T cells were transfected with ( $\pm$ Nod DNA (0.1 ng), NF- $\kappa$ B reporter, and a Renilla control. The cells were treated with stimuli for 12h, harvested and tested for luciferase activity.

The *in vitro* interaction between Nod2 and MDP is dependent on pH (Figure 6), with the pH range from 5.0 to 6.5 giving maximal binding. The data suggest that *in vivo* binding could occur in an acidic cellular compartment, a model supported by cellular assays that show pH-dependent internalization of MDP<sup>29,30</sup>. Girardin and coworkers show that the internalization of MDP is optimal in the pH range of 5.5 to 6.5, which corresponds with the MDP/Nod2 binding data.

Nod2 is predicted to have ATP binding capabilities, as it has Walker A and Walker B regions<sup>31</sup>. The ATP binding capacities of the protein have been suggested to be important for

oligomerization, protein-protein interactions and subsequent activation<sup>32</sup>. To test if the nucleotide binding was necessary for Nod2 to bind to MDP, we measured Nod2 binding to MDP  $\pm$ ATP/ADP. Nod2 binds with no appreciable change to MDP in the presence and absence of 10  $\mu$ M ATP/ADP (Figure 6), suggesting that ATP/ADP is not necessary for Nod2 to bind to MDP.

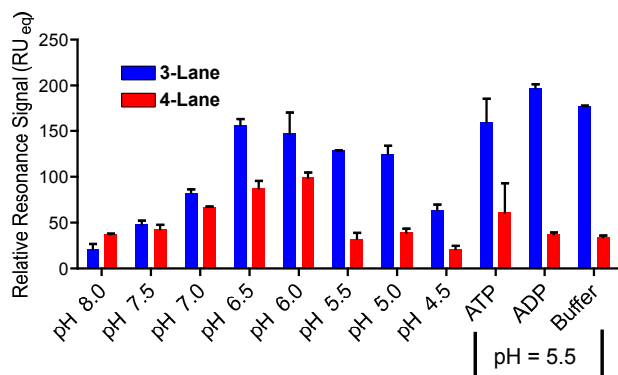


Figure 6: Nod2/MDPs interact under a variety of conditions. The pH of Nod2 was adjusted before application to the MDP-Chip. Nod2 was pre-incubated with 10  $\mu$ M ATP or ADP before application to the MDP-Chip. The relative resonance signal was recorded after ten minutes.

Prior to our investigation, the mechanism of Nod2 activation of NF- $\kappa$ B by treatment with MDP was unclear. We have taken a biochemical approach to demonstrate that Nod2 binds directly to bacterial cell wall fragments. Recombinant Nod2 and the synthetic MDP tools allowed for the development of *in vitro* assay to detect binding. The assay that we have developed will be a valuable asset in screening for inhibitors/activators of the Nod2 signaling pathway and determining if Nod2 is able to differentiate commensal versus pathogenic bacteria. In addition, the assay will be useful in determining the Nod2 Crohn's mutants are capable of binding to MDP. This is the first biochemical evidence to show an interaction between the two molecules, and establishes that MDP is a high-affinity ligand for Nod2.

## ASSOCIATED CONTENT

**Supporting Information.** Nod2 expression and purification conditions, SPR assay, and NF- $\kappa$ B assay conditions: this material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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## ABBREVIATIONS

MDP, muramyl dipeptide; SPR, surface Plasmon resonance; SAMs, self assembled monolayers (SAMs)

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